N-deoxyribosyl transferases of *Lactobacillus fermentum* and use for the enzymatic synthesis of 2',3'-dideoxynucleosides and 2',3'-didehydro-2',3'-dideoxynucleosides

The present invention relates to novel N-deoxyribosyl transferases of *Lactobacillus fermentum* and their use for the enzymatic synthesis of 2',3'-dideoxynucleosides and 2',3'-didehydro-2',3'-dideoxynucleosides.

Nucleoside analogues are very widely used in antiviral therapies or in anti-cancer chemotherapy. For example there can be mentioned ddI (didanosine), ddC (zalcitabine) and d4T (stavudine) or AZT (zidovudine) in anti-HIV therapy, ACV (acyclovir) in the treatment of herpes or also GCV (ganciclovir) used in anti-tumor therapy in combination with herpes thymidine kinase.

The dideoxynucleosides such as ddI and ddC and their derivatives are the most effective inhibitors known at present used in therapy against the HIV virus.

The chemical synthesis of these compounds requires several stages of protections, deprotections and purifications. It would therefore be desirable to be able to simplify the procedures for synthesis of this type of compounds by developing selective enzymatic and highly specific methods.

The N-deoxyribosyl transferases produced by bacteria of the *Lactobacillus* genus are enzymes which catalyze the transfer of deoxyribose between two puric or pyrimidic bases. They are also capable in general of transferring 2',3'-dideoxyribose between these same bases (Carson and Wasson, 1988). Thus, it has been possible to synthesize several pyrazolo (3,4-d) pyrimidines and triazolo (4,5-d) pyrimidines derived from 2',3'-dideoxycytidine and the corresponding base from enzymes of *Lactobacillus leichmannii* and *Lactobacillus helveticus* (Fischer et al., 1990). The 2',3'-dideoxyribose transfer reaction is however clearly less effective than that carried out with 2'-deoxyribose.

It has been found within the framework of the present invention that the introduction of mutations into N-deoxyribosyl transferase of *Lactobacillus fermentum* (*L. fermentum*), followed by a confrontation with an analogue of the natural substrate within selective screening made it possible to obtain a mutated protein having a strong activity on the novel substrate. By repeating

these operations, it appeared possible to obtain enzymes having an activity on substrates more and more distant from the initial natural substrate.

It was after a stage of random mutagenesis in an *ntd* gene of *L. fermentum*, followed by a selection stage using a functional genetic screen that it has been possible to isolate mutants having a more significant specific activity, in particular for the transfer of 2',3'-dideoxyribose.

This method for selecting more active modified enzymes more particularly involves as genetic screen the *E. coli* strain PAK 9 (deposited at the CNCM on 27th June 2002 under accession number 1-2902), which is of genotype Δ*pyrC*::Gm, Δ*codA*::Km, *cdd*::*Tn*10.

This strain makes it possible to select the production of uracil as it is deleted for the *pyrC* gene which controls the conversion of carbamyl aspartate to dihydroorotate as well as for the *codA* and *cdd* genes which control respectively the deamination of cytosine and (deoxy)cytidine. It therefore has a requirement for uracil (u) which can only be satisfied by the introduction of uridine (R-U), deoxyuracil (dR-U) or dideoxyuracil (ddR-U). However the use of dideoxyuracil (ddR-U) can be selected from the strain PAK9 only if a variant of N-deoxyribosyl transferase is capable of producing one of the following two reactions:

i) ddR-U →U + ddR,

ii) ddR-U ⇆ C= ddR-C + U

The transforming clones of PAK 9 expressing a randomly mutated *ntd* gene of L. *fermentum*, were thus selected in glucose mineral medium to which dideoxyuracil (ddR-U) and cytosine (C) have been added. Several transforming clones were obtained and are capable of carrying out the exchange

ddR-Pyr + Pur ⇆ ddR-Pur + Pyr

as well as

dR-Pyr + Pur ⇆ dR-Pur + Pyr.

The nucleotide sequences of the different variants of *ntd* of *L. fermentum* can differ from the wild-type gene only by a single mutation. Their enzymatic activities were evaluated from crude extracts or purified proteins. The specific

activity of NTD* can be 10 times less than that of NTD for the transfer of deoxyribose but can be 7 times more for the transfer of dideoxyribose.

The selected enzyme is used in the enzymatic synthesis of 2',3'-dideoxynucleosides and 2',3'-didehydro-2',3'-dideoxynucleosides of natural or modified bases (5-halogenopyrimidines), comprising or not comprising radioelements. The method can be extended to the selection of variants capable of transferring derivatives of 2'-deoxyribose or 2',3'-dideoxyribose between bases (such as 3'-amino-2',3'dideoxyribose or 3'-azido-2',3'-dideoxyribose).

Moreover, in the method according to the invention, cells in which a metabolic pathway has been inactivated can be used. The selective screening consists of making up for this deficiency by producing the product P for which the cells are auxotrophic from an analogue of the natural substrate of the protein X.

Alternatively, it is possible to evolve a protein X by complementation of a related protein Y, X and Y both belonging to the same EC enzyme nomenclature class or to adjacent classes.

DESCRIPTION

Thus, the present invention generally relates to a method of *in vitro* and *in vivo* artificial evolution of an X protein encoded by an *ntd* gene of *L. fermentum*, said method making it possible to evolve said X protein *in vivo* by complementation either of a related protein, or by complementation of an inactivated metabolic route.

Such a method makes it possible to evolve an X protein encoded by an *ntd* gene of L. *fermentum* so as to modify its characteristics by the following stages:

a) obtaining mutants of the *ntd* gene of *L. fermentum* by random mutagenesis;

b) transformation of cells comprising a [P-] phenotype with vectors comprising the mutated nucleic acid obtained in stage a) coding for the X* proteins thus modified, P- meaning that said cells are auxotrophic for the

substance P, P being the product of the action of X on its natural substrate S;

c) culture of said cells in a medium comprising a substrate S*,
S* being an analogue of the natural substrate S of said X protein;

d) selection of the cells [P-:: X*] which have survived stage c) in which the X* proteins are capable of carrying out the biosynthesis of the product P from the substrate S*.

The mutant X* protein obtained is a protein possessing an activity similar to that of natural N-deoxyribosyl transferase X. X* thus belongs to the same enzyme classes or to enzyme classes adjacent to N-deoxyribosyl transferases having at least the first three figures of the international 4-figure EC nomenclature classes. In order to pass from one class to another, the abovementioned method can be repeated with, at each passage, the addition of an additional modification to the substrate analogue designated by S*.

By "substrate analogue ", is meant the natural substrate S of the natural X protein comprising a modification or alteration. By "modification of this substrate", is meant the addition or deletion of at least one atom, group or substituent, the modification of the spatial conformation of the substrate (isomeric, enantiomeric, diasteroisomeric). This modification can be minimal or significant from the structural point of view. In the case where it is sought to substantially modify the activity of the protein (or enzyme), the method can be repeated, further modifying the substrate S* at each new selection cycle. Little by little, the protein accumulates mutations which are responsible for the modification of its activity.

In this process, the cells used in stage b) are obtained by inactivation of at least one gene involved in the natural metabolic pathway leading to the product P.

Thus, the X* protein obtained makes up for the deficiency of the natural metabolic pathway leading to the product P in a medium provided with the substrate S*.

By "complement", is meant the deletion of the auxotrophic phenotype resulting from the inactivation of the gene or the metabolic route.

Alternatively, the cells can be cells in which the gene coding for a protein related to X has been inactivated beforehand.

By "inactivation", is meant a deletion in whole or in part, an insertion, or a mutation rendering the gene inoperative. The inactivation can also consist of a modification leading to a phenotype of the Ts (temperature-sensitive) type. In this case, the cells are cultured at temperatures not permissible during the selection phase (stages c) and d)).

Preferably, the related protein Y previously mentioned possesses at least the first three figures (2.4.2) of the international 4-figure EC nomenclature (Table 1), more particularly forms part of class EC 2.4.2.6 (N-deoxyribosyl transferases).

TABLE 1

EC Number	Name according to the international nomenclature
2.4.2.5	Nucleoside ribosyl transferase.
2.4.2.6	Nucleoside deoxyribosyl transferase
2.4.2.7	Adenine phosphoribosyl transferase
2.4.2.8	Hypoxanthine phosphoribosyl transferase.
2.4.2.9	Uracil phosphoribosyl transferase.
2.4.2.10	Orotate phosphoribosyl transferase.
2.4.2.11	Nicotinate phosphoribosyl transferase.
2.4.2.12	Nicotinamide phosphoribosyl transferase.
2.4.2.14	Amidophosphoribosyl transferase.
2.4.2.17	ATP phosphoribosyl transferase.
2.4.2.18	Anthranilate phosphoribosyl transferase.
2.4.2.20	Dioxotetrahydropyrimidine phosphoribosyl transferase.
2.4.2.21	Nicotinate-Nucleotide-dimethylbenzimidazole phosphoribosyl transferase.
2.4.2.22	Xanthine-guanine phosphoribosyl transferase.
2.4.2.29	Queuine tRNA-ribosyl transferase.
2.4.2.30	NAD (+) ADP-ribosyl transferase.
2.4.2.31	NAD (P) (+)--arginine ADP-ribosyl transferase.
2.4.2.36	NAD (+)--diphthamide ADP-ribosyl transferase.
2.4.2.37	NAD (+)--dinitrogeN-reductase ADP-D-ribosyl transferase.

Advantageously, the activity of N-deoxyribosyl transferase X on the substrate S is at least 2, 5, 10, 25, 50, 100 or 1000 times greater than its activity on the substrate S*. In parallel, the activity of the X* protein on the substrate S* is at least 5, 10, 25, 50, 100 or 1000 times greater than its activity on the substrate S.

The random mutagenesis of stage a) can be carried out either by variation of the manganese concentration during the PCR reaction, or by use of promutagenic nucleotide analogues or also by the utilization of primers comprising a random sequence. Different techniques are described in the documents US 6,323,030 (Methods for generating polynucleotides having desired characteristics by iterative selection and recombination), US 6,177,263 (Recombination of polynucleotide sequences using random or defined primers), WO 01/66798 (Random truncation and amplification of nucleic acid), and EP1205547 (DNA mutagenesis by random fragmentation and reassembly).

The cells used within the framework of the invention are prokaryotic or eukaryotic cells, preferably *E. coli*.

In a particular embodiment, the invention relates to a method as described above for evolving an N-deoxyribosyl transferase (DTP) so as to obtain an N-dideoxyribosyl transferase, characterized in that it comprises the following stages:

a) obtaining DTP* mutants of the sequence of the *ntd* gene of *L. fermentum* coding for an N-deoxyribosyl transferase (DTP) by random mutagenesis;

b) transformation of cells comprising a phenotype [N-] with vectors comprising the mutated nucleic acids obtained in Stage a) coding for the DTP* proteins, N- meaning that said cells are auxotrophic for at least one nucleoside, said nucleoside being the product of the action of DTP on its natural substrate dR-N;

c) culture of said cells in a medium comprising a ddR-N substrate;

d) selection of the [N-:: DTP*] cells which have survived Stage c) in which the DTP* proteins are capable of carrying out the transfer of the dideoxyribose (ddR) from a dideoxyribonucleoside to another nucleoside

leading to the production of the N nucleoside necessary for the survival of the cells.

By "N nucleoside", is meant a natural nucleoside, i.e. molecules constituted by a sugar linked to a heterocyclic base by an N-glycosidic bond, the bases being pyrimidines (thymine, uracil, cytosine) or purines (adenine, guanine from the usual bases). By "N-" is meant an [A-, T-, G-, C-, U-or I-] phenotype.

The NTD* enzyme obtained can be capable of recognizing and transferring a deoxyribose analogue such as dideoxyribose, but also of acting on nucleoside analogues. Thus, the analogue of substrate S* used can be an analogue of deoxyribonucleoside or didehydrodideoxyribonucleosides comprising at least one chemical modification on the base and/or on the ribose.

More particularly, the coding sequence (*ntd*) of N-deoxyribosyl transferase (DTP) of *L. fermentum* corresponds to SEQ ID No. 1.

In this process, in stage b) bacteria of the genotypes *ΔpyrC, ΔcodA, Δcdd* deficient in the metabolic pathway leading to uracil can be used. The *E. coli* strain PAK 9 deposited at the CNCM on 27th June 2002 under No. 1-2902, is particularly suited to this use.

Advantageously, the present invention aims, starting with the method described above, to obtain from the protein X encoded by *ntd* of *L. fermentum*, a mutated protein having an N-dideoxyribosyl transferase activity and/or an activity on analogues of deoxy or dideoxyribonucleoside comprising a modified base. The sequence of the thus mutated protein in general has a percentage identity greater than or equal to 70%, in particular 80%, preferentially greater than or equal to 90%, and more preferentially greater than or equal to 95% with the sequence SEQ ID No. 2. It is moreover important that certain residues of the sequence ID No. 2 be preserved so that said mutated protein has an optimum enzymatic activity. This is the case in particular with the residues Y13 (tyrosine in position 13), D77 (aspartic acid in position 77), D97 (aspartic acid in position 97), E103 (glutamic acid in position 103), M132 (methionine in position 132). Thus, certain variants can have a percentage identity with the sequence ID No. 2 comprised between 70% and 80% in the regions which are situated outside the catalytic site of the enzyme

constituted by said residues. These variants then have a sequence at least 70% identical to SEQ ID No. 2, in which the residues Y13, D77, D97, E103, M132 are preserved, preferably at least 80%.

The invention thus also consists of a protein having an activity on of deoxy- or dideoxyribonucleoside analogues, having a percentage identity with SEQ ID No. 4 equal to or greater than 70%, preferably 75%, and in order of preference, respectively 80%, 85%, 90%, 95% and 98%, and comprising a threonine residue corresponding to the mutation point A15T of SEQ ID No. 4. The correspondence between the threonine residue and the mutation point A15T of SEQ ID No. 4 is in general established by alignment of the sequence of said protein with SEQ ID No. 4 as represented in Figure 3 of the present Application.

Such a protein comprises in general, moreover, the residues corresponding to Y13, D77, D97, E103 and M132 of SEQ ID No. 4, which are necessary for good catalytic activity.

Preferably, a protein according to the invention has an N-dideoxyribosyl transferase activity, which in general manifests itself by a deoxyribose and dideoxyribose and/or didehydroribose transfer activity .

A protein as defined above in general has a catalytic activity on d4T and ddT preferably at least 50% greater than that of the native N-deoxyribosyl transferase protein of *L. fermentum* represented by SEQ ID No. 2.

This catalytic activity manifests itself in particular by a catalytic effectiveness on d4T and ddT at least 5 times, preferably at least 7 times, greater than that of the native N-deoxyribosyl transferase protein of *L. fermentum* represented by SEQ ID No. 2. The catalytic effectiveness on ddT is in general 10 times, preferably 20 times and more preferentially 50 times greater than that of the native N-deoxyribosyl transferase protein of *L. fermentum* represented by SEQ ID No. 2.

By catalytic effectiveness is meant the result of the quotient Kcat/Km, which reflects the number of times that a enzyme carries out a reaction (transformation of its substrate), compared with the number of times that said enzyme forms a complex with its substrate. Thus the more effective an enzyme is, the higher will be the value of its Kcat/Km quotient.

A particularly preferred mutated protein of the invention comprises the mutation A15T, such as for example the protein of sequence SEQ ID No. 4.

The invention also relates to a nucleic acid comprising a mutated *ntd* (NTD*) sequence coding for a mutated protein as defined previously and having a N-deoxyribosyl transferase activity and/or an activity on analogues of deoxy or dideoxyribonucleoside comprising a mutated modified base. A preferred nucleic acid of the invention comprises the sequence SEQ ID No.3, which codes for the protein corresponding to SEQ ID No. 4.

The invention also relates to an expression vector comprising a nucleic acid as defined above, in particular the sequence SEQ ID No. 3. This sequence can be fused to a promoter effective for the expression of all or part of said sequence in the eukaryotic and/or prokaryotic cells. The vector can be a plasmid capable of transforming and being maintained in *E. coli*. The vector can be maintained in the bacterium in a stable or transitory manner.

The invention also relates to a host cell comprising a vector as described previously, such as the strain of *E. coli* deposited at the CNCM on 22nd March 2004 under accession number I-3192 which comprises the vector pETLFA15T described below.

In another aspect, the invention relates to the use of an N-dideoxyribosyl transferase described above for the transfer of a dideoxyribose (ddR) from a dideoxyribonucleoside to another nucleoside, in particular in order to obtain the synthesis of 2',3'-dideoxynucleosides and 2',3'-didehydro-2',3'-dideoxynucleosides.

This enzyme obtained from the method according to the invention is particularly useful for the preparation of nucleoside analogues possessing anti-tumor properties, in particular ddI or ddC.

Thus, the invention also relates to a method for the preparation of compounds comprising a stage consisting of utilizing a mutated protein defined above.

This method is particularly advantageous for the preparation of nucleoside or nucleotide analogues useful for the treatment of cancer or infectious diseases, in particular dideoxyribonucleosides, in particular ddC or ddI and 2',3'-didehydro-2',3'-dideoxynucleosides.

Reference will be made to the legends of the figures below in the remainder of the description.

Legends

- Figure 1: Biosynthesis routes
  - Figure 1a) the "*de novo*" synthesis of DNA from simple precursors. The abbreviations used are as follows:
    ndk: nucleoside diphosphokinase
    pyrA:carbamoylphosphate synthase
    pyrB: aspartate carbamoyl transferase
    pyrC: dihydroorotase
    pyrD:dihydroorotate oxydase
    pyrE: orotate phosphoribosyl transferase
    pyrF: orotidine 5'-phosphate decarboxylase
    pyrG: CTP synthetase
    pyrH: UMP kinase
  - Figure 1 b) the conservation or recycling route which is much less costly in terms of energy and involving transfer reactions of sugar from preformed bases (originating from the hydrolytic degradation of amino acids and nucleotides). The abbreviations used are as follows (enzymes represented by their corresponding genes):
    cdd: cytidine/deoxycitidine deaminase
    cmk: CMP/dCMP kinase horylase
    codA: cytosine deaminase
    deoA: thymidine phosphorylase
    tdk: thymidine kinase
    udk: uridine/cytidine kinase
    udp: uridine phosphorylase
    upp: uridine phosphoryl transferase
    thyA: thymidylate synthase

- Figure 2: Catalytic cycle of NTD

- Figure 3: Alignment of Ntd sequences showing the residues Y(Tyr)13, D(Asp)77, D(Asp)97, E(Glu)103 and M(Met)132 forming part of the catalytic site. Lh: *Lactobacillus helveticus*; La: *Lactobacillus acidophilus*, Lj: *lactobacillus johnsonni*, Ll: *Lactobacillus leichmanni*, **Lf: *Lactobacillus* fermentum**, Lm: *leuconostoc mesenteroides*, Pro mar: *prochlorococcus marinus*

EXAMPLE 1: Enzymatic synthesis of nucleosides

The synthesis of the nucleosides in *E. coli* can be carried out according to two processes; [Agnete MUNCH-PETERSEN (1983)."Metabolism of nucleotides, nucleosides and nucleobases in microorganisms" published by Academic Press] (see Figures 1a and 1b).

Two classes of enzymes exist which catalyze the transfer of a 2-deoxyribosyl to a nitrogenous base; see hereafter and [Jane R. HANRAHAN & David W. HUTCHINSON (1992). "The enzymatic synthesis of antiviral agents". Journal of Biotechnology; vol. 23; 193-210. The latter are sometimes used for the synthesis of nucleoside analogues].

The N-deoxyribosyl transferases catalyze the cleavage of the glycosidic bonds of the 2-deoxynucleotides. They are present in certain micro-organisms which possess little or no purine and pyrimidine phosphorylase (lactobacilla for example) [6-8]. They participate in the recycling of nucleotides.

Reactions catalyzed according to the type of enzymes

Two types of enzyme have been characterized, [José HOLGUIN & Robert CARDINAUD (1975). "Trans-N-Deoxyribosylase: substrate-specific studies". European Journal of Biochemistry; vol. 54; 515-520].

Purine deoxyribosyl transferase or NTD1:

It catalyzes exclusively the reversible transfer of a sugar from a puric base (donor base) to another purine base (acceptor base).

(1): (d)R-pur_1 + pur_2 ⇆ pur_1 + (d)R-pur_2

Pyrimidine/Purine deoxyribosyl transferase or NTD II

It mostly catalyzes the transfer between purine and pyrimidine according to the following reversible equations:

(2): (d)R-pyr_1 +pyr_2 ⇆ pyr_1 + (d)R-pyr_2

(3): (d)R-pyr_1 +pur_2 ⇆ pyr_1 +(d)R-pur_2

Reaction mechanism (Figure 2)

If we keep to what is known from *Lactobacillus delbruckii*, NTD II would react according to a "ping-pong-bi-bi" mechanism which would involve two substrates and two products [Jose HOLGUIN & Robert CARDINAUD (1975). "Trans-N-Deoxyribosylase: Purification by affinity chromatography and characterisation". European Journal of Biochemistry; vol. 54; 505-514; C. DANZIN & Robert CARDINAUD (1974). "Deoxyribosyl transfer catalysis with trans-N-deoxyribosylase. Kinetic studies of purine to purine trans-N-deoxyribosylase. European Journal of Biochemistry; vol. 48; 255-252; C. DANZIN & Robert CARDINAUD (1976). "Deoxyribosyl transfer catalysis with trans-N-deoxyribosylase. Kinetic studies of purine (pyrimidine) to purine (pyrimidine) trans-N-deoxyribosylase. European Journal of Biochemistry; vol.62; 356-372].

It is assumed that the sugar of the donor nucleoside ($dBase_1$) binds to the enzyme covalently. An intramolecular reaction within this binary complex allows the cleavage of the β-glycosidic bond and the formation of a ternary complex E-deoxyribosyl-$Base_1$ followed by the release of the first product ($Base_1$). The acceptor base ($Base_2$) then binds to the binary intermediate and after intramolecular reaction on the active site of the enzyme, the second product ($dBase_2$) is released. The enzyme can then carry out another catalysis.

Physico-chemical properties

In *Lactobacillus delbruckii*, the two enzymes have a similar molecular weight (evaluated at about 100 kDa) but they differ in their thermal stability (activity observed up to 45°C for NTD I et 55°C for NTD II) and their optimum pH (6.4 for NTD I and 6.0 for NTD II).

The *ntd* gene of *Lactobacillus delbruckii* coding for NTD II with a length of 471 bp codes for the synthesis of a protein with 157 amino acids and total molecular weight of 110 kDa [William J. COOK, Steven A. SHORT & Steven E. EALICK (1990). "Crystallization & preliminary X-ray investigation of recombinant *Lactobacillus leichmanii* nucleoside 2-deoxyribosyl transferase". The Journal of Biological Chemistry; vol. 265; No. 5; 2682-2683]. The crystalline structure of the enzyme NTD II of *L. delbruckii* was determined with a resolution of 2.5 Å. This is a hexamer (trimer of dimers) constituted by six identical sub-units of 18 kDa. Each sub-unit possesses in the centre a parallel β-sheet comprising five strands of various lengths and surrounded by four α helices arranged asymmetrically. Each comprises an active site, but the six catalytic centres, approximately 20 Å distant in pairs, require the participation of the side chains of the adjacent sub-units [Shelly R. ARMSTRONG, William J. COOK, Steven A. SHORT & Steven E. EALICK (1996). "Crystal structures of nucleoside 2-deoxyribosyl transferase in native & ligand-bound forms reveal architecture of the active site". Structure; vol. 4; No. 1; 97-107]. The latter are involved in the positioning of the catalytic amino acid (glutamate 98) [David J. T. PORTER, Barbara M. MERRIL & Steven A. SHORT (1995). "Identification of the active site nucleophile nucleoside 2-deoxyribosyl transferase as glutamic acid 98". The Journal of Biological chemistry; vol. 270; No. 26; 15551-15556].

Enzymatic synthesis of nucleoside analogues

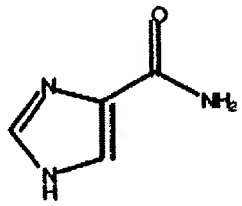
The highly stereospecific transfer reactions, in the presence of an NTD I or NTD II transferase, exclusively produce the β anomer of the nucleoside (which avoids the stage of separation of the α and β isomers).

The enzyme possesses a great specificity vis-à-vis 2'-deoxyribonucleotides but tolerates a large number of modified analogues on the sugar or on the base. Thymidine and cytosine seem to be the most effective donors of sugar. On the other hand the transfer can be made to a large panel of acceptor bases. There can for example be mentioned the purines substituted in position 6 [D. BETBEDER, D.W. HUTCHINSON & A.O. RICHARDS (1989). "The stereoselective enzymatic synthesis of 9-β-D-2',3'-

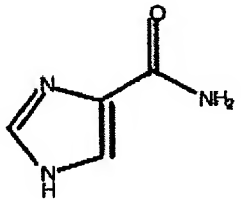
dideoxynucleosides of N(6)substituted purines". Antiviral Chem. Chemother; vol. 17; 4217-4222] and dYTP.

dYTP:

The imidazole-4-carboxamide called Y was proposed as simplified purine. This analogue has the formula:



It has been reported that the nucleotide dYTP could be substituted for dATP or dGTP during the copying of the DNA which introduces mutations. There can also be mentioned the compounds described in WO 01/96354 (Institut Pasteur) of general formula:



The NTD enzymes prove to be capable of marginally catalyzing the exchange reaction between a 2',3'-dideoxyribose and an acceptor base:

dd-1'-$Base_1$ + $Base_2$ ⇆ $Base_1$ + dd-1'-$Base_2$

(dd =2',3'-dideoxyribose)

Nevertheless the speed of this transfer remains very low compared to that characterizing the exchange of deoxyriboses.

The 2',3'-dideoxyribonucleotides are evidently useful as chain terminators in the sequencing procedures. Furthermore, 2',3'-dideoxyadenosine (ddA) and 2',3'-dideoxyinosine (ddI) are used for therapeutic purposes in particular in the case of the AIDS virus: these analogues effectively inhibit the replication of HIV (human immunodeficiency virus) [H.MITSUYA & S. BRODER (1987)."Strategies for antiviral therapy in AIDS". Nature; vol. 325; 773-778].

To this end, the invention provides a novel method for obtaining mutants of the NTD II enzyme in order to select mutant enzymes of *L. fermentum* which have a stronger specificity towards the 2',3'-dideoxyribonucleosides than the native enzyme.

EXAMPLE 2:

Application of the method according to the invention for obtaining NTD*

MATERIALS AND METHODS

The PAK9 *E. coli* strains are cultured in Luria-Bertani (LB) medium or in minimum MS medium (Richaud et al. 1993). The antibiotics kanamycin, Km and chloramphenicol Cm, are used at a final concentration of 25 µg/ml; tetracycline, Tc and gentamycin, Gm,10 µg/ml. The nucleosides and bases are used in the culture media at a final concentration of 0.3 mM. The molecular biology techniques are carried out according to Sambrook et al. (1989)

The amplification products are purified using QIAquick PCR purification (QIAgen)

The DNA fragments purified on agarose gel are extracted using the Jetsorb Kit (Genomed) or the QIAquick gel extraction kit (QIAgen). The plasmidic DNA is purified using the QIAprep spin miniprep kit (QIAgen)

The strain PAK9 (MG1655 Δ*pyrC*::Gm, Δ*codA*::Km, *cdd*::Tn10) is available from the CNCM (Collection Nationale de Culture des Microorganismes) at the Institut Pasteur, 25-28 rue du Dr Roux 75224 Paris cedex 15, under No. 1-2902.

The vector pSU19N was obtained by site-directed mutagenesis of the plasmid pSU19 [B. BARTOLOME, J. JUBETE, E. MARTINEZ & F. DE LA CRUZ (1991) "Constructions and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives" Gene; vol. 102; 75-78; E. MARTINEZ, B. BARTOLOME & F. DE LA CRUZ (1988) "pACYC184-derived cloning vectors containing the multiple cloning site and lacZ alpha reporter gene of pUC8/9 and pUC18/19 plasmids" Gene; vol 68(1); 159-162] using the oligonucleotides

PAK 23 5'P-CAATTTCACACAGGAAACACATATGACCATGATTACGCC (SEQ. ID No. 5)

PAK 24 5'P-TGTTTCCTGTGTGAAATTGTTATCCGCTCAC (SEQ. ID No. 6)

An *ntd* gene of *L. fermentum* was amplified by PCR from the plasmid pLF6 used here as DNA matrix. The plasmid pLF6 propagated from the *E. coli* strain PAK6 deposited at the CNCM on 2nd May 2001 under the reference 1-2664, contains a fragment Alu I of 1.36 kb of the gene encoding the N-deoxyribosyl transferase of type II originating from the strain *L. fermentum* CIP102780T. In order to amplify this DNA fragment, the following oligonucleotides were used:

PAK 5 5'-GATATACATATGAAAAATACCGACCCAGTTGC (SEQ. ID No. 7) and

PAK 6 5'-NNGGATCCTTAGGTTAGTTAGAAAACCTTGAATGGTGGG (SEQ. ID No. 8),

then the amplified fragments were digested by the restriction enzymes *BamH*I and *Nde*I and cloned in the vector pSU19N. In this construction, the expression of the protein is under the control of the *lac* promoter.

1) Mutagenesis

The primers T7prom (5'-TTAATACGACTCACTATAGGGG)(SEQ ID No.9) and T7term (5'-GCTAGTTATTGCTCAGCGG) (SEQ ID No.10) were used to amplify the *ntd* gene cloned in the plasmid pET24a (Novagen) according to standard amplification conditions using the GeneMorph PCR Mutagenesis Kit (Stratagene, USA). The amplification parameters: 1 cycle of 5' at 95°C, 30 cycles each comprising the following three stages: 30" at 95°C, 30" at 51.5°C,1' at 72°C, then a cycle of 10' at 72°C. The concentrations of DNA matrix used: 10 ng and 10 pg.

2) Cloning and selection

The purified amplification products are digested for 2 hours at 37°C by the restriction enzymes *BamH*I and *Nde*I. After migration at 150V for 45 minutes, they are purified by 1% agarose gel extraction using the QIAquick gel extraction kit (QIAgen).

The plasmid pSU19N is digested by the same enzymes and purified according to the same procedure.

The ligations produced in a volume of 20 µl comprise 15 ng of the amplification products, 50 ng of pSU19 digested by *BamH*I-*Hind*III, 2 µl of 10x concentrated reaction buffer of T4 DNA ligase and 6U of T4 DNA ligase. The reaction is carried out at 16°C for 18 hours.

The ligation products are then dialysed on Millipore filter (0.05 µm; 13 mm) for 30 minutes then used to transform the strain PAK9, prepared according to the protocol described by Dower et al. (1987), by electroporation.

1 to 5 µl of ligated DNA mixed with 50 µl of the strain PAK9 in a 2 mm cuvette are subjected to a charge of 2.5 kV. After incubation for one hour at 37°C in 1 ml of LB medium supplemented with uracil (0.3 mM), two successive washings with 1 ml of 1X MS medium are carried out.

450 µl of suspension are plated on mineral glucose agar medium supplemented with Cm, ddU and C. The dishes are incubated at 37°C for 4 days. The selected colonies are then isolated on the same medium.

The plasmid DNA of the isolated colonies is prepared from cultures in LB medium supplemented with Cm and U. The sequencing of the plasmids was carried out by the company MWG-BIOTECH.

The sequencing of the plasmids present in the selected transformants of PAK 9 made it possible to identify a mutation in the sequence (*ntd*) having the effect of substituting a residue T for the residue A in position 15 in the corresponding protein sequence (SEQ ID No. 2) (mutation called A15T) .

3) Measurement of the enzymatic activity of the crude extracts of the different mutants

3.1 Preparation of the crude extracts

The precultures are obtained after inoculation of an isolated colony in 5 ml of LB medium containing Cm and U for the strain PAK9 followed by incubation overnight under stirring at 37°C.

The next day, 15 ml of LB medium containing Cm and U are inoculated at an OD_{600} = 0.01. The cultures are then incubated at 37°C up to an OD comprised between 0.8 and 1.

The cells are then centrifuged at 4000 rpm for 30 minutes at 4°C, the pellet is resuspended in 10 ml of phosphate buffer (Na_2HP0_4 + NaH_2PO_4) at 50 mM (pH = 7.5). After centrifugation, the pellet is resuspended in 1 ml of the same buffer. The cells, preserved in ice, are then subjected to three cycles of 30 s of sonication and 30 s of rest. After centrifugation at 12000 rpm for 2 x 15 minutes at 4°C, the supernatants are recovered and stored at -20°C.

3.2 Enzymatic reaction

50 µl of enzymatic extract is added to 200 µl of 100 mM citrate buffer, pH 6.44, in the presence of 3 mM ddU or dU final and of 1 mM C final for the strain PAK9, the whole is incubated at 37°C. The progress of the reaction is monitored by TLC (silica; eluent: MeOH- CH_2Cl_2 (20/80)). The products are revealed under UV, and the sugars revealed by Zücker reagent. The disappearance of the substrates and the formation of the products were also quantified by HPLC analysis. The different products are separated by analytical HPLC with a reversed-phase column (100-5C18) using a flow rate of 1 ml/min and a linear gradient of 5-25% CH_3CN in a 10 mM triethyl ammonium acetate buffer at pH 7.5 for 20 minutes.

4) Overproduction and purification of the native N-deoxyribosyl transferase and the mutant LFA15T.

The oligonucleotides:

PAK 5 5'-NGATATACATATGAAAAATACCGACCCAGTTGC (SEQ ID No.11)

and

PAK 6 5'NNGGATCCTTAGGTTAGTTAGAAAACCTTGAATGGTGGG (SEQ ID No.12)

were used as primer in an amplification reaction under standard conditions using the *ntd* gene of *L. fermentum* cloned in pSU19 (pLF6) as DNA matrix. The amplification product was digested by the restriction enzymes *Nde*I and *Bam*HI for 2 hours at 37°C, purified on agarose gel and inserted into the plasmid pET24a digested by the same enzymes then the ligation mixture is used to transform the strain β 2033. The plasmid DNA from the colonies was prepared and digested by the enzymes *Nde*I and *Bam*HI. Those, the

sequence of which was correct, were used to transform the strain BL21 (DE3)/p*lys*S (Novagen). The plasmid DNA of the mutant pSU19NLFA15T selected previously was prepared then digested by the enzymes *Nde*I and *Bam*HI. The corresponding fragment *Nde*I-*Bam*HI was then inserted into the plasmid pET24a digested by the same enzymes in order to produce the expression plasmid pETLFA15T useful to the expression of the mutated protein. A strain of *E. coli* transformed using the plasmid pETLFA15T was deposited at the CNCM on 22nd March 2004 under accession number I-3192. The overproduction of the two N-deoxyribosyl transferases, native and mutated, was obtained from cultures of this strain in 500 ml of LB medium supplemented with Km and Cm. These cultures were induced at an OD_{600} = 0.6 by the addition of IPTG (0.4 mM), the incubation being continued for 2 hours 30 minutes at 37°C.

The cells are then centrifuged for 15' at 4000 rpm at 4°C, washed in 50 ml of phosphate buffer then the pellet obtained after centrifugation is preserved overnight at -20°C. The bacterial pellet resuspended in 20 ml of phosphate buffer is then lysed by passage through a French press at 14000 psi. The lysate is centrifuged for 90' at 50,000 rpm. The supernatant containing the soluble proteins is then precipitated with ammonium sulphate (40% saturation). The precipitate obtained after centrifugation at 13900 rpm (20,000g) for 30' at 4°C is resuspended in 1 ml of 100 mM phosphate buffer, pH 7.5, 1.5 M NaCl, then deposited on a Sephacryl S200 gel filtration column (Amersham-Pharmacia). The fractions are then analyzed by SDS-PAGE gel and the enzymatic activity determined. The most active and purest fractions are dialysed overnight at 4°C against the same buffer at pH = 6.0. The protein concentration is determined by measuring the OD at 280 nm.

The measurement of the enzymatic activities is carried out as described in paragraph 4.2.

5) Results

The transforming clones of the *E. coli* strain PAK9, expressing the mutated *ntd* gene of *L. fermentans* were selected in glucose mineral medium with dideoxyuracil (ddR-U) and cytosine (C) added.

Several transformants were obtained and are capable of carrying out the exchange:

ddR-Pyr + Pur ⇔ ddR-Pur + Pyr as well as dR-Pyr + Pur ⇔ dR-Pur + Pyr.

The nucleotide sequences of the different variants of *ntd* are identical and only differ from the wild-type gene by one mutation (indicated in bold type in Table 2 below). In both cases (*L. leichmannii* and *L. fermentum*) a neutral amino acid (glycine and alanine) is replaced by a nucleophilic amino acid (serine and threonine respectively). The conversion of N-deoxyribosyl transferase to N-dideoxyribosyl transferase or N-didehydroribosyl transferase therefore seems to require the substitution of a neutral amino acid by a nucleophilic amino acid which must contribute to the positioning of the sugar promoting its catalysis. It is interesting to note in Table 2 that all the N-deoxyribosyl transferases as well as a certain number of homologous proteins (of unknown function) possess a glycine or an alanine in this position.

TABLE 2

Origin of the mutated gene	Corresponding protein sequence		
NTD *Lactobacillus. acidophilus*	MMAKTKTLYF	G	AGWFNEKQNKAYKAAMEALKQN
NTD *Lactobacillus. helveticus*	MNKKKTLYF	G	AGWFNEKQNKAYKEAMAALKEN
NTD *Lactobacillus. leichmannii*	MPKKTIYF	G	AGWFTDRQNKAYKEAMEALKEN
NTD LIG9S	MPKKTIYF	**S**	AGWFTDRQNKAYKEAMEALKEN
PTD *Lactobacillus. helveticus*	MKAVVPTG-KIYL	G	SPFYSDAQRERAAKAKELLAKN
Lactobacillus gasseri	MTKQKTVYF	G	AGWFTETQNKAY
NTD *Lactobacillus. fermentum*	LKNTDPVANTKIYL	A	TSFFNEEQRARIPQALAQLEAN
NTDLFA15T	LKNTDPVANTKIYL	**T**	TSFFNEEQRARIPQALAQLEAN
Oenococcus oeni MCW	MNMAKNIYL	A	SPFFDDEQIARVKKIEKALESN
Leuconostoc mesenteroides ATCC 8293	KNVYL	A	SPFFDKEQIERVERVEKALAAN
Lactobacillus plantarum WCFS1	VYL	A	APFFDEAQKERIQQVKSALLAN
Lactococcus lactis IL1403	NQAVNVYL	A	APFFSESQIKK

The enzymatic activities of the native and mutant N-deoxyribosyl transferases of *L. leichmannii* (LL and LL G9S) and of *L. fermentum* (LF and LFA15T) in the exchange reactions dT +C⇔dC + T, ddT +C⇔ddC + T and d4T +C ⇔d4C + T were evaluated starting from crude extracts or purified proteins.

The results reported in Table 3 below show that the specific activity of the mutant LFA15T is less than that of the native enzyme (LF) for the transfer of deoxyribose but that the latter is greater for the transfer of dideoxyribose or didehydroribose. For the transfer of deoxyribose, the activity is reduced by a factor of 7, whereas the latter is increased by 3 in the case of the transfer of dideoxyribose and by 35 in the case of didehydroribose.

TABLE 3

	LL	LL G9S	LF	LFA15T
dT + C	100	10	76.5	10.7
ddT + C	0.2	2.5	0.9	2.5
d4T + C	0.5	8	2.1	73.5

Note: 100% at the top of the LL column represents the specific activity of the enzyme NTD of *L. leichmannii* during the reaction dT + C ⇔ dC + T.

Table 4 below shows in detail the results of enzymatic activity tests for the native enzyme and the mutated enzyme of *B. fermentum* for each of the dT + C, ddT + C and d4T + C reactions. The first column of the table shows the affinity constant values (Km), the second the maximum reaction speed (Vmax), the third, the catalysis constant (Kcat), and the last the ratio of the affinity and catalysis constants (Km/Kcat) taking account of the effectiveness of the enzymes tested. These different values were measured according to the protocol described in the literature [P A Kaminski (2002) "Functional cloning, heterologous expression and purification of two different N-deoxyribosyl transferases from *Lactobacillus* helveticus" J. Biol. Chem; vol. 277; 14400-14407]. The enzyme mutated according to the method of the invention shows a better catalytic activity on d4T and on ddT than the native enzyme. The activities are increased respectively by 60% and 54%. Moreover, the mutated enzyme LFA15T is 60 times more effective than the native enzyme LF in the ddT + X exchange and 7.5 times more effective in the d4T + X exchange.

TABLE 4

	Km µM	Vmax µmol/s	Kcat µmol/s/µg	Kcat/km
LF dT	124	6.65	0.665	5.36
LF ddT	80	5.7	0.038	0.047
LF d4T	1250	24	0.56	0.448
LFA15T dT	371	9.7	0.242	0.65
LFA15T ddT	53	7.8	0.156	2.9
LFA15Td4T	1.1	18.4	3.68	3.34

The selected enzyme is therefore used in the enzymatic synthesis of 2',3'-dideoxynucleosides and 2',3'-dideoxy, 2',3'-didehydronucleosides from natural bases ddC, ddA, ddI, d4T, d4C, d4G (Ray *et al.* 2002; Stuyver *et al.* 2002) or modified bases (Pokrovsky *et al.* 2001 Chong *et al.*, 2002) such as (1β-3'-fluoro) 2',3'-dideoxy, 2',3'-didehydro-4'-thio-Nucleosides comprising or not comprising radioelements.

6) Determination of the residues involved in the catalytic site of the enzyme Ntd:

As shown by the alignment of Figure 3, the residues Y(Tyr)13, D(Asp)77, D (Asp)97, E(Glu)103 and M(Met)132 (numbering established in relation to Ntd of *B. fermentum* - SEQ ID No. 2) are to be found particularly well-preserved in the Ntd proteins of the different microorganisms represented. Point mutagenesis experiments targeting these residues have made it possible to establish that they were involved in the catalytic site of the enzyme. In fact, the mutation of one of these residues results in a loss of activity of the enzyme of the order of 90%.

REFERENCES

Bartolome B, Jubete Y, Martinez E, de la Cruz F. (1991) Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. *Gene*. **102**: 75-8

Carson D.A. & Wasson D.B. (1988) Synthesis of 2',3'-dideoxynucleosides by enzymatic trans-glycosylation. *Biochem. Biophys. Res. Comm.* **155**: 829-834.

Chong Y, Choo H, Choi Y, Mathew J, Schinazi RF, Chu CK. Stereoselective synthesis and antiviral activity of D-2',3'-didehydro-2',3'-dideoxy-2'-fluoro-4'-thionucleosides. *J Med Chem.* 2002 **45**: 4888-98.

Dower WJ, Miller JF, Ragsdale CW. (1988) "High efficiency transformation of *E. coli* by high voltage electroporation." *Nucleic Acids Res.* **16**: 6127-45.

Fischer, X., Kaun, E. and Genz, U. (1990) 2',3'-Dideoxyribofuranosides and method for their production. Ger. Offen. DE 3840160.

Pokrovsky AG, Pronayeva TR, Fedyuk NV, Shirokova EA, Khandazhinskaya AL, Tarusova NB, Karpenko IL, Krayevsky AA. (2001) Anti-HIV activity of novel phosphonate derivatives of AZT, d4T, and ddA. *Nucleosides Nucleotides Nucleic Acids*. **4-7**: 767-9.

Ray AS, Yang Z, Chu CK, Anderson KS. Novel use of a guanosine prodrug approach to convert 2',3'-didehydro-2',3'-dideoxyguanosine into a viable antiviral agent. *Antimicrob Agents Chemother*. 2002 **46**: 887-91.

Richaud C, Mengi N, Lecreulx D, Pochet S, Johnson EJ, Cohen GN, Marliere P. (1993) Directed evolution of biosynthetic pathways. Recruitment of cysteine thioethers for constructing the cell wall of *Escherichia coli*. *J Biol Chem*. **268**: 26827-35.

Secrist JA 3rd, Riggs RM, Tiwari KN, Montgomery JA. Synthesis and anti-HIV activity of 4'-thio-2',3'-dideoxynucleosides. *J Med Chem* 1992 **35**: 533-8

Stuyver LJ, Lostia S, Adams M, Mathew JS, Pai BS, Grier J, Tharnish PM, Choi Y, Chong Y, Choo H, Chu CK, Otto MJ, Schinazi RF. Antiviral activities and cellular toxicities of modified 2',3'-dideoxy- 2',3'-didehydrocytidine analogues. *Antimicrob Agents Chemother*. 2002 **46**: 3854-60.

Van Draanen NA, Freeman GA, Short SA, Harvey R, Jansen R, Szczech G, Koszalka GW. (1996) "Synthesis and antiviral activity of 2'-deoxy-4'-thio purine nucleosides." *J Med Chem* **39**: 538-42